Increased susceptibility to bacterial superinfection as a consequence of innate antiviral responses

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The reason why severe localized or systemic virus infections enhance and aggravate bacterial superinfection is poorly understood. Here we show that virus-induced IFN type I caused apoptosis in bone marrow granulocytes, drastically reduced granulocyte infiltrates at the site of bacterial superinfection, caused up to 1,000-fold higher bacterial titers in solid organs, and increased disease susceptibility. The finding that the innate antiviral immune response reduces the antibacterial granulocyte defense offers an explanation for enhanced susceptibility to bacterial superinfection during viral disease.

bacteria | granulocytopenia | IFN I | virus infection

Better mechanistical insight into the process of facilitated bacterial superinfection during virus infection is needed because it is responsible for many of the 200,000 annual cases of life-endangering sepsis in the United States alone (1). Although several local-acting mechanisms have been proposed (2–5), an explanation for generalized enhanced susceptibility to superinfection is still lacking. To address this issue, we have modeled a systemic bacterial superinfection during virus infection by combining well studied model infections in mice. Lymphocytic choriomeningitis virus (LCMV) is a noncytopathic RNA virus that rapidly spreads systemically and strongly induces type I IFN (IFN I) (6). *Listeria monocytogenes* (L.m.) and *Staphylococcus aureus* are Gram-positive bacteria that cause purulent infections. Both pathogens induce strong innate immune responses and attract granulocytes (7) and subsequently T cell-dependent macrophages, whose role is to clear the bacteria by phagocytosis (8–10). During neutropenia caused by cytotoxic drugs, irradiation, or administration of monoclonal antibodies, hosts are rendered much more susceptible to bacterial infections (11–13). Here we show that during virus infection IFN I has an early severe granulotoxic effect that drastically increases susceptibility to bacterial superinfection.

Results

Increased Susceptibility to *Listeria* Superinfection Early After LCMV Infection. When C57BL/6 mice were infected with LCMV-WE 2 days before superinfection with L.m. (Fig. 1*A*), they exhibited up to 1,000-fold-higher bacterial titers in liver and spleen at day 3 of the superinfection compared with control mice without LCMV infection (Fig. 1*B*) as well as drastically decreased survival (Fig. 1*C*). This early phase of L.m. infection is largely controlled by innate resistance through granulocytes (12–16), whereas, after day 3, T cell-mediated control is crucial (17). Failure to control bacterial superinfection was not simply a consequence of virus replication, because bacterial elimination was also enhanced at day 5 after LCMV infection when virus titers were comparable to or even higher than those at day 3 (Fig. 5, which is published as supporting information on the PNAS web site, and data not shown). Together with existing evidence, this finding suggested that the underlying mechanism reflected a transient change of the early host antibacterial response. This finding was akin to the observation of an unexplained susceptibility to bacterial superinfection in the lung in the timeframe of days 2–5 after influenza A virus infection (3). In humans, bacterial superinfections become clinically apparent variably but usually starting as soon as 3–5 days after the onset of virus-induced clinical symptoms (1). The recognized major role of granulocytes in controlling early L.m. infection (9, 11–13) was confirmed by depletion of granulocytes at day 1 after bacterial infection by administration of an anti-GR1 antibody (Fig. 1*B*). Thus, increased susceptibility due to viral preinfection could be mimicked by the physical absence of granulocytes. To determine whether granulocytes were indeed involved and affected, bone marrow granulocytes were quantified by FACS 3 days after superinfection. Granulocytes were strongly reduced in LCMV-preinfected mice compared with control mice up to day 5 (Fig. 1*D*).

Effects of LCMV Infection on Granulocytes. Next, granulocyte kinetics after LCMV infection in the absence of bacterial superinfection were measured. Granulocyte numbers in bone marrow of C57BL/6 mice were analyzed at different time points after LCMV-WE infection (Fig. 1*E*). After an initial small increase, granulocyte numbers dropped to ≈30% of the original number 2–5 days after LCMV infection. This observation correlated with induction of granulocyte apoptosis (Fig. 1*E*). Thereafter, granulocyte numbers normalized by day 9. A study analyzing influenza infection in monkeys and one study of human patients revealed a reduction of blood polymorphonuclear leukocytes at day 3 after admission to the hospital (18, 19). The fate of other cell types during LCMV infection was also investigated by immunohistology. In spleen, macrophages increased somewhat at day 5 after LCMV infection, whereas liver macrophages remained unchanged during the entire period analyzed (Fig. 6*A*, which is published as supporting information on the PNAs web site). Natural killer cell numbers in the bone marrow stayed at low levels until day 5, then increased in numbers (Fig. 6*B*). T cell numbers were low until day 7, when the expected LCMV-associated T cell proliferation could be demonstrated (Fig. 6*B*). Early during LCMV infection $B220^+$ B cells decreased (20) comparably to granulocytes but did not recover to original numbers during the 15 days studied, reflecting the known

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The authors declare no conflict of interest.

Abbreviations: LCMV, lymphocytic choriomeningitis virus; L.m., *Listeria monocytogenes*; 7-AAD, 7-amino-actinomycin D.

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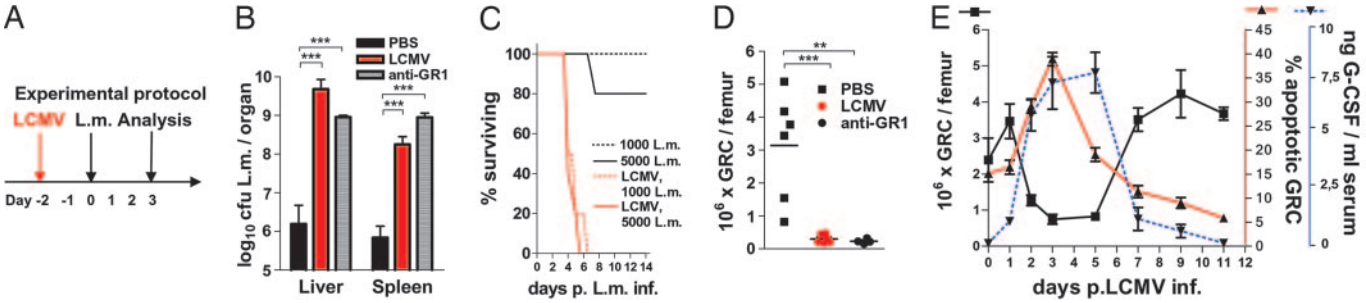

Fig. 1. Increased susceptibility to bacterial infection early during LCMV-induced bone marrow granulocytopenia. (*A*) Experimental protocol. (*B*) Bacterial titers in spleen and liver at day 3 of infection. C57BL/6 mice were infected with 2×10^6 pfu of LCMV-WE i.v. 48 h before infection with 5×10^3 cfu (1/2 LD_{50}) of L.m. i.v. For comparison, 120 μg of granulocyte-depleting antibody anti-GR1 NimpR14 was given i.v. at day 1 after L.m. infection in an additional group of mice (mean ± SEM of four to six animals per group; one of three experiments). (*C*) C57BL/6 mice were infected with LCMV at day −2 and infected with 1×10^3 (1/10 LD_{50}) or 5×10^3 (1/2 LD_{50}) cfu of L.m. at day 0. Survival was monitored twice daily ($n = 9$–10 per group). (*D*) Bone marrow granulocyte (GRC and $Ly6G^+CD11b^+$) cell numbers measured by FACS at day 5 after LCMV and day 3 after L.m. superinfection ($n = 4$–6 animals per group; one of three experiments). (*E*) Bone marrow GRC (black) of C57BL/6 mice after infection with 2×10^6 pfu of LCMV-WE i.v. Shown are the percentage of annexin V^+ of GRC (red) (mean ± SEM; $n = 3$–4 per group; one of two experiments with similar results) and the granulocyte colony-stimulating factor concentration in serum (blue) (mean ± SEM; $n = 3$–4 per group).

immunopathological destruction of lymphoid tissue (21) (Fig. 6*B*). Thus, only granulocyte kinetics correlated with the relatively short time period of virus-induced failure to control bacterial superinfection.

To determine whether virus-induced granulocytopenia might be associated with excessive activation, expression of the β_2-integrin CD11b on the surface of bone marrow granulocytes after virus infection was measured (Fig. 2*A*). Granulocytes were activated by 24 h after LCMV infection, and by day 7 CD11b surface expression had normalized. Both phagocytosis and superoxide production by bone marrow granulocytes were increased in LCMV-infected compared with untreated animals (Fig. 2 *B* and *C*). To determine whether the observed state of preactivation resulted in heightened cell damage upon contact with live bacteria, we measured the percentage of 7-aminoactinomycin D (7-AAD)-positive granulocytes after 1 h of coincubation with L.m. Dead granulocytes were increased 2.5-fold after virus infection and bacterial challenge vs. untreated, whereas both virus and bacterial infection alone also induced granulocyte damage (1.7- and 1.9-fold of untreated) (Figs. 1*E* and 2*D*).

Analysis of Parameters of LCMV-Induced Granulocytopenia. Our results so far demonstrated that granulocyte activation and induction of apoptosis correlated with a failure to effectively counter bacterial superinfection. Mechanistically, the lowered granulocyte reservoir in the bone marrow could limit the effective granulocyte homing to the site of bacterial superinfection.

C57BL/6 mice were challenged with a high dose of L.m. at day 3 of LCMV infection. Numbers of infiltrating granulocytes were analyzed in spleen and liver 4 h after L.m. injection. Virus-infected animals showed 3-fold fewer granulocytes in both organs (Fig. 2*E*). In an additional experiment, thioglycolate, known to provoke rapid recruitment of granulocytes into the peritoneum (22), was injected i.p. at day 3 of LCMV infection. Four hours later, LCMV-infected mice demonstrated 50% fewer granulocytes in the peritoneal cavity than control animals (Fig. 2*F*).

The observed bone marrow granulocytopenia could reflect several mechanisms, reduction by apoptosis in the bone marrow and/or emigration and accumulation in the periphery. The third possibility, i.e., reduced induction of granulopoiesis, was an unlikely explanation because strongly elevated expression of granulocyte colony-stimulating factor at the time of granulocyte depletion was measured (Fig. 1*E*). Assessment of apoptosis on bone marrow cell suspensions after LCMV infection revealed increased granulocyte apoptosis starting from day 2 after infection. This observation inversely correlated with the number of granulocytes present in the bone marrow (Fig. 1*E*). Induction of apoptosis was specific for granulocytes, because nongranulocytic

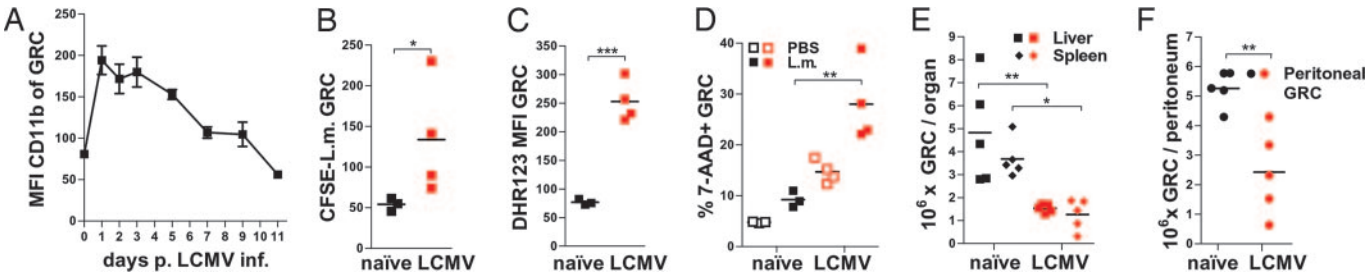

Fig. 2. Activation-associated cell death of bone marrow GRC during LCMV infection leads to impaired granulocytic infiltration. (*A*) Up-regulation of CD11b surface expression on bone marrow GRC during LCMV infection ($n = 3$–4 animals per time point; mean fluorescence intensity ± SEM). (*B*) Phagocytosis of CFSE-labeled L.m. *in vitro* by bone marrow GRC (multiplicity of infection 20:1) at day 2 after LCMV infection after 1 h of coincubation. Shown is the mean fluorescence intensity of 7-AAD-negative GRC (one of two experiments with similar results). (*C*) NADPH oxidase activity in 7-AAD-negative GRC. Shown is bone marrow GRC as measured by dihydrorhodamine 123 fluorescence (534 nm) *in vitro* upon coincubation with L.m. for 1 h at day 2 after LCMV infection (one of three experiments with similar results). (*D*) Percentage of dead (7-AAD-positive) bone marrow GRC from LCMV-preinfected C57BL/6 mice or control mice after incubation with or without L.m. *in vitro* for 1 h (one of two experiments with similar results). (*E*) Numbers of hepatic and splenic GRC 4 h after L.m. challenge (10^7 cfu i.v.) in C57BL/6 mice at day 3 after 2×10^6 pfu of LCMV infection (one of two experiments with similar results). (*F*) Numbers of peritoneal GRC 4 h after administration of 1 ml of thioglycolate i.p. in C57BL/6 mice at day 3 after LCMV infection (one of two experiments with similar results).

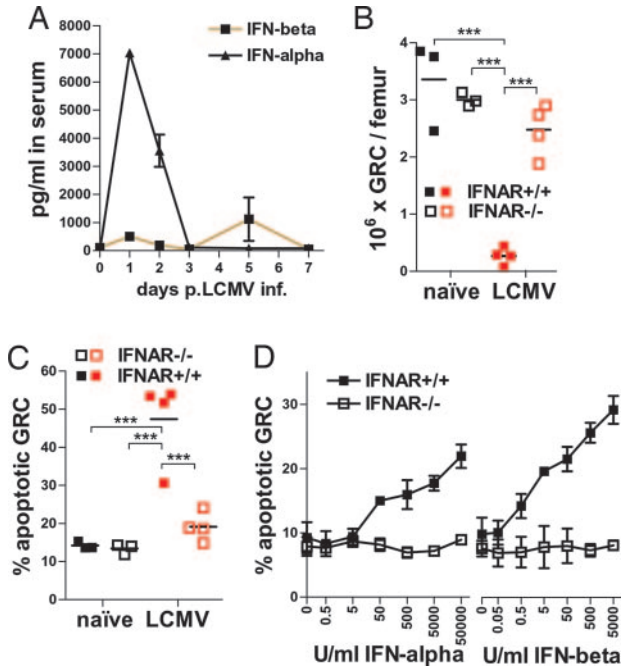

Fig. 3. IFN I-dependent bone marrow GRC apoptosis. (*A*) Serum IFN I in LCMV-infected C57BL/6 mice (n = 3–4; mean ± SEM). (*B*) Numbers of bone marrow GRC of IFNAR$^{-/-}$ and control mice at day 3 after LCMV infection or without virus infection (one of two experiments with similar results). (*C*) Percentages of apoptotic (annexin V$^+$) bone marrow GRC of IFNAR$^{-/-}$ and control mice at day 3 after LCMV infection or without virus infection (n = 3–4 animals per group; mean ± SEM; one of two experiments with similar results). (*D*) Apoptotic GRC (Ly6G$^+$) after 72 h of *in vitro* bone marrow culture of wild-type and IFNAR$^{-/-}$ mice with increasing amounts of recombinant IFN-α or IFN-β (one of two similar experiments).

bone marrow cells did not show elevated apoptosis at day 3, the time point of maximal granulocyte apoptosis (Fig. 6*C*). We then tested whether granulocytes showed enhanced emigration and accumulation in the periphery by quantifying granulocytes in blood, spleen, and liver by FACS and immunohistology (Fig. 6*E*). Blood granulocyte numbers dropped early during virus infection, then stayed low and increased only after day 5 (Fig. 6*D*). In spleen, detectable granulocytes approximately doubled by day 2 after LCMV infection, corresponding to approximately two million granulocytes per spleen. Because we observed a loss of ≈70% of the total 65 million granulocytes (7) in the entire bone marrow, enhanced migration could not explain bone marrow granulocyte loss. Granulocyte numbers remained unchanged in the liver (Fig. 6*E*). Taken together, activation-associated granulocyte death after virus infection was the main mechanism for the observed bone marrow granulocytopenia.

IFN I Effects on Granulocytes. Next we tested whether granulocyte apoptosis was a consequence of antiviral mediators produced early during LCMV infection. IFN-α production after LCMV infection peaked at day 1 after infection and inversely predicted bone marrow granulocyte numbers (Fig. 3*A*). To demonstrate a causal relationship, IFN I receptor knockout mice (IFNAR$^{-/-}$) were infected with LCMV, and bone marrow granulocyte numbers were measured at day 3 when T cell responses had not been mounted yet and viral titers were equal in both strains (23). IFNAR$^{-/-}$ mice did not exhibit a reduction of granulocytes in the bone marrow whereas wild-type mice had 12-fold-reduced granulocyte numbers compared with untreated animals (Fig. 3*B*). Furthermore, IFNAR$^{-/-}$ mice showed no increase in granulocyte apoptosis after LCMV infection *in vivo* (Fig. 3*C*). Correspondingly, bone marrow cell suspensions of naïve wild-type but not IFNAR$^{-/-}$ treated *in vitro* with recombinant IFN I for 72 h revealed a dose-dependent induction of granulocyte apoptosis (Fig. 3*D*).

To confirm the crucial role of virus-induced IFN I in impairing granulocyte-mediated control of bacterial infection, IFNAR$^{-/-}$ and wild-type mice were LCMV-infected and 2 days later were superinfected with L.m. Another 2 days later, wild-type mice displayed 1,000-fold-higher bacterial titers than IFNAR$^{-/-}$ in spleen and ≈20-fold-higher titers in liver (Fig. 4*A*). This finding correlated with drastically lower granulocyte infiltration in the periphery in LCMV-infected wild-type mice (Fig. 4 *C* and *D* and Fig. 7 *Bottom*, which is published as supporting information on the PNAS web site). The virus-induced bone marrow granulocytopenia was not complete but became functionally limiting during bacterial superinfection, where large numbers of bone marrow-derived granulocytes are required to control bacterial lesions. During these experiments, granulocyte numbers were also monitored in blood. Whereas naïve mice and LCMV-preinfected IFNAR$^{-/-}$ mice animals increased granulocytes in blood during L.m. infection, LCMV-infected wild-type animals were unable to mount reactive granulocytosis upon superinfection (Fig. 4*B*).

Recent reports have implicated a detrimental effect of IFN I during L.m. infection partly by acting on T cells (24–27). Although at the dose of L.m. and in the 129/SvEv background we were using these L.m. titer differences were marginal, our findings pointed in the same direction as the results of these recent studies. The Toll-like receptor 3 ligand poly(I:C) has been described to increase susceptibility to L.m. infection (24). We therefore analyzed whether poly(I:C) affects the granulocyte compartment similar to a LCMV preinfection. IFNAR-competent and -deficient mice were infected with 5×10^3 cfu of L.m. and treated with 200 μg of poly(I:C) at the day of infection. As described, this treatment resulted in increased L.m. titers only in IFNAR-expressing animals at day 3 of the L.m. infection (Fig. 8*A*, which is published as supporting information on the PNAS web site). When bone marrow granulocytes were analyzed, poly(I:C)-treated wild-type animals showed increased activation (Fig. 8*B*) and apoptosis (Fig. 8*C*) within the granulocyte compartment that was reduced in numbers (Fig. 8 *D–F*). Poly(I:C) treatment alone also resulted in IFNAR-dependent reduction of granulocyte numbers and increased apoptosis in the bone marrow (Fig. 8 *G* and *H*).

Enhancement by LCMV Infection of Susceptibility to Various Bacterial Superinfections. To determine whether susceptibility to superinfection extended to other bacteria, the usually extracellular bacterium *S. aureus* was analyzed. IFNAR$^{-/-}$ mice were preinfected with LCMV and challenged 2 days later with *S. aureus*. After 24 h, LCMV-infected wild-type mice demonstrated drastic clinical disease as well as 100-fold-higher bacterial titers in lung and kidney when compared with IFNAR$^{-/-}$ mice (Fig. 4*E*). LCMV-infected C57BL/6 mice were also tested to exclude mouse strain differences: they revealed 100-fold-increased bacterial organ titers after infection (data not shown). To determine whether virus-induced granulocytopenia would also heighten susceptibility to a Gram-negative superinfection, we tested *Salmonella typhimurium* in C57BL/6 mice (11). At day 2 of LCMV infection, 1.2×10^4 cfu of *Sa. typhimurium* were given i.v. Bacterial titers were significantly elevated at day 3 in spleen and liver in LCMV-preinfected mice compared with non-pretreated mice (Fig. 4*F*).

To test whether bacterial superinfection at the onset of bone marrow granulocyte recovery after LCMV infection would be cleared normally, we infected C57BL/6 mice with 5,000 L.m. at day 5 after LCMV. Interestingly, and perhaps as might have been

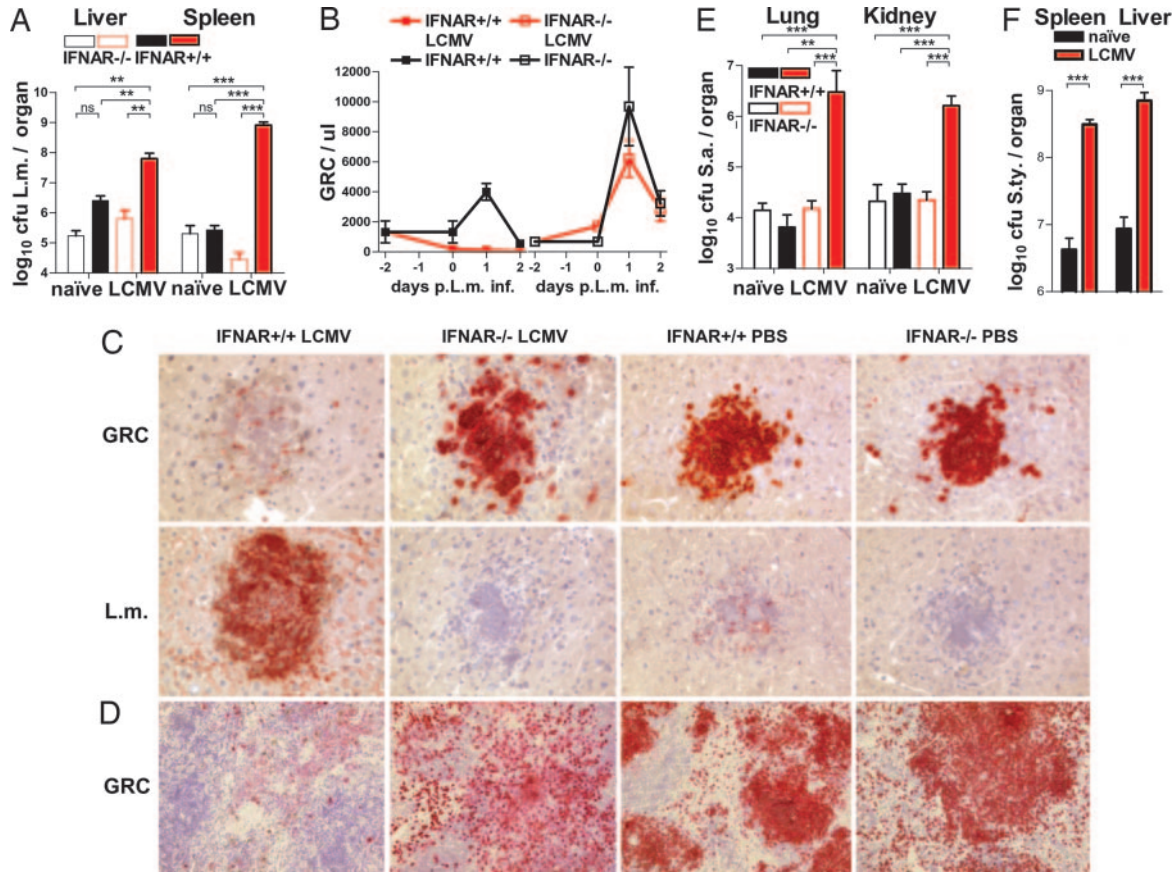

Fig. 4. IFN I-dependent granulocytopenia and absence of granulocyte infiltrates at the site of bacterial infection. (*A*) IFNAR$^{-/-}$ mice and wild-type mice were infected with LCMV 48 h before superinfection with 5×10^3 L.m. Two days later, L.m. titers were determined (n = 3–4 per group; one of three comparable experiments is shown; mean ± SEM). (*B*) Numbers of peripheral blood GRC (n = 3–4 per group; one of two experiments; mean ± SEM). (*C* and *D*) Immunohistochemistry of GRC (GR1) in liver (*C Upper*) and spleen (*D*) and of L.m. (rabbit anti-L.m.; *C Lower*) in sequential sections at day 2 after superinfection with L.m. in LCMV-infected IFNAR$^{-/-}$ or control wild-type mice (two experiments with similar results). (*E*) IFNAR$^{-/-}$ mice and control mice were infected with LCMV at day −2 and superinfected at day 0 with 2×10^7 cfu of *S. aureus*. Twenty-four hours later, *S. aureus* titers were determined (n = 3–4 per group; mean ± SEM). (*F*) C57BL/6 mice infected with LCMV at day −2 were superinfected with 1.2×10^4 *Sa. typhimurium* i.v., and bacterial titers were determined at day 3.

expected at this later time point, mice cleared these infections better than controls.

Discussion

The virus-induced suppression of antibacterial resistance and immunity by IFN I production was caused by apoptosis of bone marrow granulocytes and impaired granulocyte emigration to sites of bacterial infection. This granulocytopenia was not complete but became functionally limiting during superinfection, where large numbers of bone marrow-derived granulocytes were rapidly required to control L.m., *S. aureus*, or *Sa. typhimurium*. Because LCMV is not cytopathic in its murine host, direct cell destruction by replicating virus is not necessary for enhancement of bacterial superinfection.

In addition to systemic effects, local virus-induced alterations including enzymatic uncovering of bacterial binding sites on host cells (5), lysis of epithelial cells by cytopathic viruses (4), which may break antibacterial barriers (2), and enhanced bacterial binding to virus-encoded receptors that are expressed on infected host cells (3) may additionally facilitate bacterial superinfection. IFN-induced granulocyte apoptosis could also explain sepsis often observed after burn injuries or ischemic stroke (28–30). From the point of view of the virus, the induction of innate antiviral IFN I, by depleting granulocytes, may also diminish later adaptive immune responses (31–33) and may thus favor virus persistence. Neutropenia is a regular symptom of virus infections such as EBV (34) and HIV (35). IFN I as a therapeutic agent has also been shown to induce granulocytopenia (36, 37), and hepatitis C and B patients receiving therapeutic IFN I have been shown to have an increased risk for bacterial superinfections when rendered neutropenic by treatment (38, 39).

Taken together, our findings suggest that virus-induced granulocytopenia may critically contribute to bacterial superinfections. Attempts to block this effect, possibly with preventive granulocyte colony-stimulating factor application or perhaps even cautious anti-IFN I treatments during the acute phase of the virus infection, could ameliorate the outcome of the described clinical constellation.

Materials and Methods

Mice. Specific-pathogen-free laboratory mice were from the Institute of Labortierkunde of the veterinary facility of the University of Zurich (Zurich, Switzerland). Experiments were performed according to Swiss veterinary law and institutional guidelines. C57BL/6 mice, IFN I receptor (IFNAR) knockout mice, and relevant controls on 129/SvEv background were used.

Infections. L.m. strain 10403S was grown overnight in brain–heart infusion broth, washed two times in PBS, and injected i.v. in 200 μl; bacterial counts were monitored as indicated in *Bacterial Titer* and are detailed in each experiment. LCMV strain WE (from F.

Lehmann-Grube, Heinrich Pette Institute, Hamburg, Germany) was grown on BHK cells, and 2×10^6 pfu were injected i.v. in 200 μl. *S. aureus* Newman ATCC 25904 (*S. aureus*) was grown in brain–heart infusion medium, washed two times, and frozen down. Thawed aliquots washed and then used for infection were given i.v. in 200 μl at a dose of 2×10^7 cfu per mouse. *Sa. typhimurium* was grown in LB medium and given i.v. in 200 μl at a dose of 1.2×10^4 per mouse.

Bacterial Titer. Bacterial titers were determined in homogenized halves of spleen, the left lobe of the liver, the left lung, or the whole left kidney in PBS with serial dilutions plated on brain–heart infusion or blood agar plates.

Bone Marrow Aspirates and Culture. Bone marrow cells were flushed with PBS from the femur and stained for FACS analysis or cultured in RPMI medium 1640 with 10% FCS.

FACS and Antibodies. Anti-Ly6G, GR1, CD11b, annexin V, and 7-AAD were obtained from Becton Dickinson (Basel, Switzerland). Cells expressing Ly6G/GR1 and CD11b are termed "GRC." Forward-scatter and side-scatter gates were used to exclude debris in organ homogenates and peritoneal washings. Dihydrorhodamine 123 (Sigma, St. Louis, MO) was used for measuring NADPH oxidase activity by measuring cellular fluorescence in FL1 channel (emission 534 nm). A fixed number of fluorescent allophycocyanin beads were used to quantify cell number per sample volume as described in ref. 25. Anti-GR1 (NimpR14) hybridoma was a generous gift from F. Tacchini-Cottier (World Health Organization, Geneva, Switzerland) (26).

Histology. Histological samples were snap-frozen in Hanks' medium and stained with an anti-L.m. rabbit serum, Gr-1 (Pharmingen, San Jose, CA), or F4/80 (BM8 and BMA) antibodies. Staining was developed by using a goat anti-rat antibody (Caltag Laboratories, Burlingame, CA) or goat anti-rabbit (Jackson ImmunoResearch, West Grove, PA) and an alkaline phosphatase-coupled donkey anti-goat antibody (Jackson ImmunoResearch) with naphthol 6-bromo-2-hydroxy-3-naphtholic acid 2-methoxy anilide phosphate and new fuchsin as a substrate. The presence of alkaline phosphatase activity yielded a red reaction product. The sections were counterstained with hemalum.

Determination of Cytokine Production. ELISAs for murine IFN-α and IFN-β by PBL Biomedical Laboratories (Piscataway, NJ) were used on sera and culture supernatants. Granulocyte colony-stimulating factor was measured with an ELISA from R & D Systems (Minneapolis, MN).

Statistical Analysis. In all figures, an unpaired two-sided *t* test or one-way ANOVA was used. *P* values >0.05 were not considered significant (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

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